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(54) ANTIBODY RECOGNIZING ONLY VITEILOGENIN OF ORYZIAS LATIPES

(57)Abstract:
PROBLEM TO BE SOLVED: To obtain an antibody recognizing only a vitellogenin of Oryzias latipes and to provide a method for assaying a toxicity and an environmental pollution of chemical substances with using the antibody and a method for discriminating the sex of Oryzias latipes because a generally used method for an immunoassay does not exist and a method for assaying endocrine disrupting actions with using derivation of the vitellogenin as an index has not established yet.
SOLUTION: This antibody recognizes only the vitellogenin of Oryzias latipes. A method for assaying the concentration of the vitellogenin of Oryzias latipes is characterized by reacting the antibody with the vitellogenin in the humor of Oryzias latipes.

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CLAIMS

[Claim(s)]
[Claim 1] The antibody which recognizes only BITEROJIENIN of a cyprinodont.
[Claim 2] The antibody which recognizes only BITEROJIENIN of the cyprinodont according to claim 1 said whose antibody is either a monoclonal antibody or a polyclonal antibody.
[Claim 3] A trust number is FERM. Antibody which recognizes only BITEROJIENIN of a cyprinodont produced by the hybridoma which is P-17665.
[Claim 4] The density measurement approach of BITEROJIENIN of a cyprinodont characterized by making an antibody and BITEROJIENIN in the body fluid of a cyprinodont according to claim 1 to 3 react.
[Claim 5] How to make an antibody and BITEROJIENIN in the body fluid of a cyprinodont according to claim 1 to 3 react, and to evaluate the toxicity of a chemical, or environmental pollution by making into an index the rise of the concentration of BITEROJIENIN measured by this reaction.
[Claim 6] How to evaluate the toxicity of a chemical according to claim 5 or environmental pollution whose chemical is what has an endocrine disruption operation.
[Claim 7] How to evaluate the toxicity of a chemical according to claim 5 or environmental pollution which is what environmental pollution depends on the chemical which has an endocrine disruption operation.
[Claim 8] The sex judging approach of the cyprinodont characterized by making an antibody and BITEROJIENIN in the body fluid of a cyprinodont according to claim 1 to 3 react.
[Claim 9] The toxicity containing an antibody according to claim 1 to 3 of a chemical, or the kit for evaluation of environmental pollution.
[Claim 10] The kit for a sex judging containing an antibody according to claim 1 to 3 of a cyprinodont.

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DETAILED DESCRIPTION

[Detailed Description of the Invention]
[0001]
[Field of the Invention] This invention relates to the toxicity of the antibody which recognizes only BITEROJIENIN of a cyprinodont, and the chemical which used this antibody for the list or the evaluation approach of environmental pollution, and the sex judging approach of a cyprinodont.
[0002]
[Description of the Prior Art] The abnormalities in reproduction of the animal considered that current and a chemical are the causes are observed in every corner of the earth. For example, the report of the abnormalities in reproduction in the United States of America and the APOPUKA lake of Florida is known best. According to the investigation report, many individuals which shrank from the magnitude of 1 / 4 - 1/2 with the normal penis of a male alligator were discovered, it compared normally in one of these, and a female alligator, and formation of the multi-**** follicle of the ovary and a polykaryotic egg was remarkable. It became clear that the cause was what is depended on the matter called JIKOHORU which is the DDT related substance taken out from the chemical plant located by the lake, and it has turned out that JIKOHORU is what has the same operation as a female sex hormone.
[0003] thus, the chemical which acts on the endocrine system of a living thing and carries out the failure of the function -- endocrine disruptors (endcrine disruptors) -- it is called the so-called environmental hormone and known as matter in which an organochlorine compound, bisphenol A, an organometallic compound, etc. have an endocrine disruption operation until now. And it is suspected whether the matter with the operation same also in various strange compounds other than these matter exists.
[0004] The chemical with structure similar to the estrogen compound which is an original female sex hormone is that association to the receptor in the living body is shown, and causing a certain kind of hormone action is known. The example also caused many abnormalities in a reproductive organ in the child born to 1950- 1970 from the mother who took DES (synthetic hormone drug) in the U.S.
[0005] However, the chemical mentioned above was not necessarily structure similar to estrogen in many cases, and it was very difficult to predict as endocrine disruptors.
[0006] Conventionally, as an approach of evaluating an endocrine disruption operation of a chemical, yeast is made to discover a estrogen acceptor in a test tube, a mouse etc. is medicated with a chemical in the approach of measuring the degree which the compound concerned combines with the acceptor, or in the living body, and the approach of analyzing the generative function etc. is adopted. However, in the test method in the former test tube, effect of the metabolite of a chemical in the living body cannot be evaluated. Moreover, in a test method in the living body [latter], that use a lot of animals for observation and analysis, and the analysis takes a great effort etc. also has many problems.
[0007] By the way, if fishes etc. are bred under existence of the chemical which has an endocrine disruption operation, it is known that BITEROJIENIN which is a yolk protein precursor peculiar to a female will be guided also into a male living body (Steroids 1980 Mar;35(3):315-328 and Le Menn F et al.). Therefore, the attempt which is going to evaluate the endocrine disruption operation which a chemical has has been made by measuring the concentration of BITEROJIENIN. However, since BITEROJIENIN was the unstable matter, the big problem was to measure correctly depending on instrumental analyses, such as high performance chromatography. Moreover, there is no approach used general-purpose also about immunological measurement, and the evaluation approach of the endocrine disruption operation which made induction of BITEROJIENIN the index is not yet established.
[0008]

[Problem(s) to be Solved by the Invention] This invention aims at offering the toxicity of the antibody which recognizes only BITEROJIENIN of a cyprinodont, and the chemical which used this antibody for the list or the evaluation approach of environmental pollution, and the sex judging approach of a cyprinodont.

[0009]

[Means for Solving the Problem] By using as an antigen BITEROJIENIN refined from the inside of cyprinodont blood, as a result of inquiring wholeheartedly, in order to solve the above-mentioned technical problem, this invention persons succeed in producing the antibody which does not react to other related protein originating in the ovary which exists in body fluid, but reacts only to BITEROJIENIN specifically, and came to complete this invention.

[0010] That is, this invention is an antibody which recognizes only BITEROJIENIN of a cyprinodont. This antibody is either a monoclonal antibody or a polyclonal antibody, and a trust number is FERM. What is produced by the hybridoma which is P-17665 is mentioned.

[0011] Furthermore, this invention is the approach of evaluating the toxicity of a chemical or environmental pollution characterized by making said monoclonal or polyclonal antibody, and BITEROJIENIN in the body fluid of a cyprinodont reacting. As a chemical, what causes an endocrine disruption operation, for example is mentioned, and what is depended on the endocrine disruption operation which the chemical concerned produces owing to as environmental pollution is mentioned.

[0012] A cyprinodont (Cyprinodonidae) is the department of one of Atheriniformes, and lives [in the fresh water of a temperate district] from a tropical area and is short-lived. It is called the environmental indicator animal that it is easy to be influenced of environmental, and is suitable as an object which evaluates the toxicity of a chemical, or environmental pollution. Furthermore, since he is comparatively able to be several cm even if the length is large, and to produce in large quantities from several mm, the industrial use to environment assessment is possible for a cyprinodont.

[0013] Furthermore, this invention is the sex judging approach of the cyprinodont characterized by making said monoclonal or polyclonal antibody, and BITEROJIENIN in the body fluid of a cyprinodont react.

[0014] Furthermore, this invention is a kit for evaluating the toxicity of a chemical or environmental pollution containing said monoclonal or polyclonal antibody.

[0015] Hereafter, this invention is explained to a detail.

[0016]

[Embodiment of the Invention] This inventions are simple and a thing which makes it possible to measure to high sensitivity about BITEROJIENIN by making this antibody react with BITEROJIENIN in the body fluid of a cyprinodont about the monoclonal or polyclonal antibody which can recognize specifically BITEROJIENIN which is yolk protein peculiar to the female of a cyprinodont. Consequently, this invention estimates the toxicity (especially reinforcement of an endocrine disruption operation) of a chemical, the contamination situation of the endocrine disruptors in a certain area is grasped, and it becomes possible to distinguish the sex of a cyprinodont further.

[0017] By carrying out immunity of the animal by using as an antigen BITEROJIENIN purely refined from the cyprinodont blood serum, with other constituents of BITEROJIENIN which separates and exists in body fluid, this invention persons did not react but thought that the monoclonal or polyclonal antibody which recognizes BITEROJIENIN specifically was obtained. I thought that measurement of BITEROJIENIN concentration was attained correctly, without being influenced of other related protein which originates in the ovary which exists in body fluid by this. Moreover, this invention persons thought that the monoclonal or polyclonal antibody which recognizes BITEROJIENIN of a cyprinodont specifically could be obtained by using BITEROJIENIN of two or more fishes for screening. So, in this invention, while producing the antibody which recognizes only BITEROJIENIN of a cyprinodont, using this antibody, it is simple and exact and the measuring method of high sensitivity BITEROJIENIN is established compared with a high speed liquid chromatography etc. And the toxic (endocrine disruption operation) reinforcement which a chemical has using the antibody of this invention is examined. This reinforcement can be evaluated by measuring whether BITEROJIENIN concentration in the living body rises intentionally with the chemical which has an endocrine disruption operation.

[0018] Furthermore, this invention person thought that sex distinction of a cyprinodont was possible by making BITEROJIENIN concentration into an index. So, in this invention, the sex distinguishing method

of a cyprinodont is established by making the above-mentioned monoclonal or a polyclonal antibody react with BITEROJIENIN, and measuring BITEROJIENIN concentration.
[0019] 1. In order to carry out immunity of the animal in producing the monoclonal or polyclonal antibody of preparation this invention of BITEROJIENIN, and in order to choose the hybridoma which recognizes BITEROJIENIN of a cyprinodont specifically, it is necessary to prepare pure BITEROJIENIN. This protein is the following, and can be made and prepared.
[0020] (1) Preparation BITEROJIENIN of BITEROJIENIN is the precursor of the phosphoprotein contained in the yolk, is made from liver in large quantities, and is secreted in blood. In this invention, a blood serum can be prepared being able to cover the blood extracted from the cyprinodont over centrifugal separation, and BITEROJIENIN can be refined by combining centrifugal separation, a gel-filtration column chromatography, etc. suitably further.
[0021] Refined BITEROJIENIN is used for the immunity for producing the antibody which recognizes BITEROJIENIN specifically. Moreover, it is used for screening of the hybridoma which produces the monoclonal antibody which recognizes BITEROJIENIN of a cyprinodont specifically etc.
[0022] 2. Although the monoclonal which recognizes only BITEROJIENIN of a cyprinodont, the monoclonal of production this invention of a polyclonal antibody, or a polyclonal antibody usually means the whole antibody molecule which can be combined with BITEROJIENIN of a cyprinodont, as long as it can combine with BITEROJIENIN of a cyprinodont, it may be the fragment (for example, Fab or F(ab')2 fragment).
[0023] The monoclonal or polyclonal antibody of this invention can be manufactured by various approaches. The manufacturing method of such an antibody is [[which is common knowledge in the field concerned], for example, Sambrook, J et al., Molecular Cloning, and Cold Spring Harbor Laboratory Press (1989) Reference]
[0024] (1) Medicate mammalian, for example, a rat, a mouse, a rabbit, etc. by using as an antigen BITEROJIENIN of the cyprinodont which is the extraction above of an antibody forming cell, and was made and prepared. The dose per animal of an antigen is 0.1-100mg, when not using an adjuvant, and when using an adjuvant, it is 1-100microg. As an adjuvant, the Freund's complete adjuvant (FCA), the Freund's incomplete adjuvant (FIA), hydroxylation aluminium adjuvant, etc. are mentioned. Immunity is mainly performed by pouring into hypodermically, intraperitoneal, etc. in a vein. moreover, especially spacing of immunity is limited -- not having -- several week spacing from several -- it is two - five-week spacing preferably, and immunity is performed 2 to 5 times preferably 1 to 10 times. And an antibody forming cell is preferably collected one - 14 days after one - 60 days after the last immunity day. As an antibody forming cell, although a spleen cell, a lymph gland cell, a peripheral blood cell, etc. are mentioned, a spleen cell or a partial lymph gland cell is desirable.
[0025] (2) In order to obtain a cell fusion hybridoma, perform the cell fusion of an antibody forming cell and a myeloma cell. Generally [animals, such as a mouse,] as a myeloma cell united with an antibody forming cell, an available established cell line can be used. As for the cell strain to be used, what has the drugs selectivity which can survive only in the condition of could not survive in the HAT selective medium (hypoxanthine, aminopterin, and thymidine being included) in the state of un-uniting, but having united with the antibody forming cell is desirable. As a myeloma cell, mouse myeloma cell strains, such as P3X63-Ag.8.U1 (P3U1) and NS-I, are mentioned, for example.
[0026] Next, the cell fusion of the above-mentioned myeloma cell and the antibody forming cell is carried out. Cell fusion mixes 1x106 to 2.5x106 antibody forming cells/ml, and 2x105 to 2x106 myeloma [/ml] cells in culture media for animal cell culture, such as DMEM which does not contain a blood serum, and RPMI-1640 culture medium, (the cell ratio 5:1 of an antibody forming cell and a myeloma cell is desirable), and performs a fusion reaction under cell fusion accelerator existence. As a cell fusion accelerator, a polyethylene glycol with a mean molecular weight of 1000-6000dalton etc. can be used. Moreover, an antibody forming cell and a myeloma cell can also be united using the cell fusion equipment of marketing using electrical stimulation (for example, electroporation).
[0027] (3) Sort out the hybridoma made into the purpose from the cell after sorting of a hybridoma, and cloning cell fusion processing. as the approach -- cell suspension -- for example, fetal-calf-serum content RPMI-1640 culture medium etc. -- suitable -- after dilution and a microtiter plate top -- 3x105 pieces / well extent firewood -- each -- a selective medium is added to a well and it cultivates by exchanging selective media suitably henceforth. Consequently, the cell grown after culture initiation and

from around the 14th by the selective medium can be obtained as a hybridoma.
[0028] Next, it screens whether the target antibody exists in the culture supernatant of the increased hybridoma. Screening of a hybridoma is not limited especially that what is necessary is just to follow the usual approach. For example, a part of culture supernatant contained in the well grown as a hybridoma can be collected, and it can screen with enzyme immunoassay, radioimmunoassay, etc.
[0029] Cloning of syncytium is performed by limiting dilution etc. and establishes the hybridoma which is finally a monoclonal antibody production cell.
[0030] 3C1 and 3H5 are obtained as a hybridoma which produces the monoclonal antibody of this invention as mentioned above. Among these, 3C1 calls "Mouse-Mousehybridoma 3C1", and is deposited with National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology (1-1-3, Higashi, Tsukuba-shi, Ibaraki-ken) as FERMP-17665 on November 29, Heisei 11.
[0031] (4) As an approach of extracting a monoclonal antibody, a usual cell culture method or the usual ascites forming method etc. is employable from the hybridoma in which the monoclonal antibody carried out extraction establishment.
[0032] In a cell culture method, a hybridoma is cultivated for seven - 14 days by the usual culture condition (for example, 37 degrees C, 5%CO2 concentration) in animal cell culture culture media, such as 10% fetal-calf-serum content RPMI-1640 culture medium, an MEM culture medium, or a serum free medium, and an antibody is acquired from the culture supernatant.
[0033] In the case of the ascites forming method, intraperitoneal [of the mammalian of the myeloma cell origin and an of-the-same-kind system animal] is medicated with about 1x107 hybridomas, and it proliferates a hybridoma in large quantities. And ascites is collected after one - two weeks.
[0034] When purification of an antibody is needed in the extraction approach of the above-mentioned antibody, it can refine by choosing suitably well-known approaches, such as an ammonium-sulfate salting-out method, an ion exchange chromatography, gel filtration, and affinity chromatography, or combining these.
[0035] Although the monoclonal or polyclonal antibody of this invention reacts specifically with BITEROJIENIN of a cyprinodont, BITEROJIENIN of other kinds, such as a carp, a red sea bream, and MAMICHOGU, has the property in which it does not react.
[0036] Moreover, the subtype of the monoclonal antibody of this invention is IgG1 as a result of a commercial typing kit's investigating.
[0037] 3. In density measurement approach this invention of BITEROJIENIN, concentration of BITEROJIENIN of the body fluid (for example, plasma, a blood serum, cyprinodont grinding liquid, etc.) of a cyprinodont can be measured using said monoclonal or polyclonal antibody (quantum).
[0038] For example, after coating a plate with the blood serum of a cyprinodont, said monoclonal or polyclonal antibody is added and is made to react. The anti-mouse IgG antibody which carried out the horseradish peroxidase (HRP) indicator further is combined with the monoclonal or polyclonal antibody of this invention on a plate after washing a plate. Then, the BITEROJIENIN concentration in a sample can be measured by making a chromophoric substrate add and color and measuring an absorbance.
[0039] 4. In the toxicity of a chemical, or evaluation this invention of environmental pollution, the toxicity of a chemical or environmental pollution can be evaluated by making into an index the rise of the concentration of said BITEROJIENIN measured by carrying out like 3.
[0040] For example, after breeding a male cyprinodont under existence of the chemical used as the candidate for evaluation, extract blood etc., BITEROJIENIN contained in a sample is made to react with the monoclonal of this invention, or a polyclonal antibody, and BITEROJIENIN concentration is measured. The size of the obtained measured value can estimate the toxicity (for example, reinforcement of an endocrine disruption operation) of a chemical. That is, a male cyprinodont is bred under existence of the chemical (for example, PCB, bisphenol A, phthalic ester) used as the candidate for evaluation, the BITEROJIENIN concentration in a blood serum is measured with time, and it compares with the BITEROJIENIN concentration in the blood serum obtained from the cyprinodont of contrast. Consequently, the chemical with which BITEROJIENIN concentration serves as a candidate for evaluation when high as compared with contrast is judged to have an endocrine disruption operation.
[0041] Moreover, it is possible by measuring the BITEROJIENIN concentration when breeding under the estrogen existence of a certain concentration, and the BITEROJIENIN concentration when breeding under the chemical existence used as the candidate for evaluation to relativize the reinforcement of an

endocrine disruption operation of the chemical used as the candidate for evaluation with estrogen, and to evaluate it. That is, rather than BITEROJIENIN concentration when the BITEROJIENIN concentration when breeding under the estrogen existence of a certain concentration (for example, 100 ppm) breeds under existence of the chemical the reinforcement of an endocrine disruption operation is unknown and is [chemical] estrogen and this concentration (for example, 100 ppm), when it is 1/10, an endocrine disruption operation of the chemical concerned can be judged to be 10 times stronger to estrogen.
[0042] In addition, BITEROJIENIN concentration can be measured by said approach of 3.
[0043] 5. In approach this invention which evaluates the environmental pollution condition by the chemical which has an endocrine disruption operation, the contamination condition of the environment by the chemical which has an endocrine disruption operation can be evaluated by making BITEROJIENIN in the body fluid of the cyprinodont of the male which inhabits the river used as the candidate for evaluation, a lake, etc. react with the monoclonal of this invention, or a polyclonal antibody, and measuring the concentration of BITEROJIENIN. The measurement result can judge that the environment is polluted with the chemical which has a certain endocrine disruption operation, when high as compared with contrast.
[0044] In addition, BITEROJIENIN concentration can be measured by said approach of 3.
[0045] 6. In approach this invention which distinguishes the sex of a cyprinodont, it is possible by making an aforementioned monoclonal or an aforementioned polyclonal antibody react with BITEROJIENIN in the body fluid of a cyprinodont, and measuring BITEROJIENIN concentration to distinguish the sex of a cyprinodont. For example, the blood of a cyprinodont is extracted, and if can measure the BITEROJIENIN concentration, it cannot be detected and a male and concentration are high, it can be judged as a female.
[0046] In addition, BITEROJIENIN concentration can be measured by said approach of 3.
[0047] However, in order to carry out the sex judging of a cyprinodont, it is required to use the cyprinodont captured from a laboratory or a nursery etc. which is not polluted with a chemical.
[0048] 7. The monoclonal or polyclonal antibody of kit this invention for a sex judging of the toxicity of a chemical, the object for evaluation of environmental pollution, or a cyprinodont can be diluted with the well-known buffer solution (pH 7-8) of 1M-10M, can be made into 0.01 microg/ml - 5microg [/ml] concentration, and can be used as the toxicity of a chemical, the object for evaluation of environmental pollution, or a kit for a sex judging of a cyprinodont. As the buffer solution, a phosphate buffer solution, the McLaren buffer solution, tris buffers, veronal buffer solution, the glycine buffer solution, etc. are mentioned.
[0049] Moreover, the monoclonal or polyclonal antibody of this invention can also be fixed and used for support. Sepharose etc. is mentioned as support. For example, it can be used by combining the monoclonal or polyclonal antibody of this invention with support, and making it distribute in the buffer solution, or stuffing a column.
[0050]
[Example] Hereafter, an example explains this invention still more concretely. However, as for this invention, the technical range is not limited to these examples.
[0051] [Example 1] The ascites extracted from the cyprinodont individual which carried out preparation (i) BITEROJIENIN 17beta-estradiol processing of the preparation (1) antigen of a monoclonal antibody was mixed with 20mM tris buffers (pH8.0), and it added in H2 column (46x100mm) which equilibrated with this buffer solution. It was eluted in a part for 5ml/of the rates of flow by the gradient method of 20mM tris buffers (pH8.0) and 1.5M sodium chloride content-20mM tris buffers (pH8.0). The peak acquired 5 minutes after adding the ascites sample was made into purification BITEROJIENIN.
[0052] (2) Immunity 0.5 of an animal After mixing the mg/ml antigen (BITEROJIENIN) with the equivalent Freund adjuvant and producing an emulsion, immunity was carried out the animal 200micro everyl. /into the back hide of a Balb/c mouse. The booster (200microl./(animal)) was performed every two weeks, after carrying out immunity 3 times from a priming, it collected blood from the eye socket, and antibody titer was checked.
[0053] (3) the PBS solution containing cyprinodont BITEROJIENIN with a measurement [of antibody titer] of 5microg [/ml] -- every [100microl/well] -- in addition to the ELISA plate, at 4 degrees C, it put overnight and solid-phase-ized. Tween-PBS containing 5mg [/ml] BSA was added 200microl/well, and at the room temperature, it put for 1 hour and blocked. Then, the antiserum diluted with Tween-

PBS containing 1mg [/ml] BSA was added 100microl/well, and was made to react at a room temperature for 2 hours. Furthermore, the HRP indicator anti-mouse IgG goat antibody diluted with Tween-PBS containing 1mg [/ml] BSA was added 100microg/well, and was made to react at a room temperature for 1 hour.

[0054] Next, 2mg/ml OPD (o-phenylenediamine dihydrochloride) was added 100microl/well, the coloring reaction was performed for 3 minutes at the room temperature, and the absorbance of 490nm was measured with the microplate reader.

[0055] (4) The cell fusion of the spleen cell of the mouse with which the rise of the cloning antibody titer of cell fusion and an antibody production hybridoma was checked, and myeloma cell P3U1 was carried out by the polyethylene-glycol method at a rate of 5:1, and selective culture of a hybridoma was performed by the HAT selective medium. Hybridoma culture supernatants were collected on the 10th day of the cell fusion, ELISA was performed by the technique of having measured antibody titer, and the same technique, and the stock whose reactivity with cyprinodont BITEROJIENIN is a positivity was screened.

[0056] After measuring the number of cells of a hybridoma which became a positivity by the above-mentioned screening, it wound around 96 well plate and was crowded so that it might be set to one piece / well (subcloning), and only the well of a single colony screened again ten days after. Similarly subcloning was again performed about the hybridoma which became a positivity by screening, and screening actuation was continued until the property was in agreement 100%.

[0057] Consequently, two shares (3C1, 3H5) of specific antibody forming cells were obtained to cyprinodont BITEROJIENIN. Moreover, the subtype of the monoclonal antibody of this invention checked that it was IgG1 with the commercial typing kit.

[0058] In addition, hybridoma 3C1 (name : "Mouse-Mouse hybridoma 3c1") is FERM on November 29, Heisei 11 to National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology (1-1-3, Higashi, Tsukuba-shi, Ibaraki-ken). It ****s as P-17665.

[0059] [Example 2] Monoclonal antibody production cell strain 3C1 of this invention which recognizes only examination cyprinodont BITEROJIENIN about species-specific [of the monoclonal antibody of this invention], and 3H5 were examined about species-specific.

[0060] BITEROJIENIN of a cyprinodont used as an antigen was refined from the plasma of Metz. Moreover, the carp was checked by the blood serum of Metz again with the plasma of the male which processed a red sea bream, MAMICHOGU, and BITEROJIENIN of a carp by 17beta-estradiol.

[0061] the PBS solution containing BITEROJIENIN of each 5microg [/ml] fish stock -- every [100microl/well] -- in addition to the ELISA plate, at 4 degrees C, it put overnight and solid-phase-ized. Tween-PBS containing 5mg [/ml] BSA was added 200microl/well, and at the room temperature, it put for 1 hour and blocked. Then, the antiserum diluted with Tween-PBS containing 1mg [/ml] BSA was added 100microl/well, and was made to react at a room temperature for 2 hours. Furthermore, the HRP indicator anti-mouse IgG goat antibody diluted with Tween-PBS containing 1mg [/ml] BSA was added 100microg/well, and was made to react at a room temperature for 1 hour. Next, 2mg/ml OPD (o-phenylenediamine dihydrochloride) was added 100microl/well, the coloring reaction was performed for 3 minutes at the room temperature, and the absorbance of 490nm was measured with the microplate reader.

[0062] Consequently, it checked that, as for BITEROJIENIN of other kinds, the monoclonal antibody of this invention did not show reactivity although BITEROJIENIN of a cyprinodont reacts (drawing 1).

[0063] Moreover, electrophoresis was performed for the plasma obtained from various kinds of fish by acrylamide gel 7%, and the protein on gel was imprinted to the nitrocellulose membrane. The imprinted nitrocellulose membrane was shaken at the room temperature in the block ace (Snow Brand Milk Products) for 1 hour. Then, the nitrocellulose membrane was dipped in the antibody solution (3C1) diluted with the dilution block ace 5 times, and it shook at the room temperature for 2 hours. The nitrocellulose membrane was shaken for 10 minutes to the Tween content PBS (T-PBS) 0.05%, and the film was washed. Furthermore, T-PBS was exchanged and same washing actuation was performed twice. The nitrocellulose membrane was dipped in the HRP indicator anti-mouse IgG goat antibody solution diluted with the dilution block ace 5000 times 5 times after membranous washing, and it shook at the room temperature for 1 hour. Then, washing actuation of the film was performed like the above. After being the tris sodium chloride buffer solution and rinsing a nitrocellulose membrane, the antigen-

antibody reaction was detected using the ECL detection system (Amersham).
[0064] Consequently, it checked that, as for BITEROJIENIN of other kinds, BITEROJIENIN of a cyprinodont did not show reactivity although the monoclonal antibody of this invention reacts also in this approach (drawing 2). Therefore, it turned out that two sorts of monoclonal antibody production cells of this invention are hybridomas which produce a specific antibody to BITEROJIENIN of a cyprinodont.
[0065] [Example 3] In order to measure BITEROJIENIN in a sample using the monoclonal antibody of examination this invention of the system of measurement of BITEROJIENIN, the sandwiches ELISA method was applied.
[0066] namely, the phosphate buffer solution (PBS) solution containing the 5microg [/ml] monoclonal antibody of this invention produced by hybridoma 3C1 -- every [50microl/well] -- it put in and coated with 4 degrees C overnight. After removing an antibody solution, it blocked for 1 hour by T-PBS which contains BSA 0.5% after 3 times washing by Tween-PBS (T-PBS) 0.05%. after washing and 1mg/ml purification cyprinodont BITEROJIENIN which carried out phase dilution by Tween-PBS containing BSA -- every [50microl/well] -- it put in and was made to react at a room temperature for 2 hours a 2microg/mlHRP indicator [after repeating the same washing actuation as the above 3 times] anti-cyprinodont BITEROJIENIN rabbit IgG antibody -- every [50microl/well] -- in addition, it reacted at the room temperature for 1 hour.
[0067] the citrate buffer solution (pH5.0) which contains 2mg [/ml] OPD (o-phenylenediamine) after repeating the same washing actuation as the above 5 times -- every [100microl/well] -- it put in and was made to react for 15 minutes After the reaction, 50microl/well addition of the 2-N sulfuric acid was carried out, and the stop and the absorbance of 490nm were measured for the reaction.
[0068] Consequently, in this system of measurement, a measurement limitation is 4 ng(s)/ml, and it was checked that it is possible to measure BITEROJIENIN of a cyprinodont to high sensitivity (drawing 3).
[0069] [Example 4] Measurement HIMEDAKA of the BITEROJIENIN concentration in a blood serum of the cyprinodont which exposed endocrine disruptors into the tank was divided into the group which was exposed by every two groups into the tank, and exposed 17beta-estradiol by the concentration of 5 ppm into these tanks as ten males and a trial group, and the group which is not exposed.
[0070] The blood serum was separated from HIMEDAKA of exposure initiation and two weeks after, and BITEROJIENIN in a blood serum was detected with the electrophoresis method given [each group] in an example 2. Consequently, in the group which exposed 17beta-estradiol, the band of strong BITEROJIENIN was detectable (drawing 4).
[0071] [Example 5] Estradiol is dissolved in the measurement result tank of whenever [effect / of the living body on an endocrine system disturbance chemical] so that it may become the concentration of 0, 0.03, 0.1, 0.3, and 1ppb. After breeding 5-6 male cyprinodonts at a time for two weeks by each concentration and being exposed, the blood was extracted, and when measured by the system of measurement which shows the blood serum after centrifugal processing to an example 2, as shown in the following table, BITEROJIENIN concentration also rose depending on estradiol concentration. It became clear that whenever [effect / of / from this / the living body on an endocrine system disturbance chemical which has the estrogen's activity by this system of measurement] could be measured.
[Table 1]

エストラジオール濃度 (ppb)	雄メダカ個体数	ビテロジェニン濃度平均 (μg/ml)
0	6	N. D
0. 1	6	693
0. 3	6	9, 021
1	5	87, 700

N.D It is [0072] below a measurement limitation.
[Example 6] Explanation of the ELISA kit for cyprinodont Vg measurement, and operation (the contents of a kit)

(1) An antibody solid phase-ized antibody microplate An one-sheet (2) cyprinodont Vg reference standard 300micro Lx2(3) specimen diluent 30mL(s)x2(4) biotin-ized anti-cyprinodont Vg antibody (x100) 70microHRP[Lx1(5)]-streptoavidin (x500) 20micro Lx1(6) substrate liquid 15mLx1(7) OPD (alt.phenylenediamine) 2 lock (8) concentration penetrant remover (x20) 30mLx1(9) reaction stop solution 6mLx1[0073] (Measurement principle) The sandwiches ELISA method using an antibody specific to Cyprinodont Vg (refer to drawing 5)

[0074] (Operation)

(1) Take out only the well used from a reagent preparation (b) antibody solid phase-ized microplate aluminum sheet, and put a penetrant remover for 10 - 60 minutes at 300microL / well ****, and a room temperature.

(b) The standard-solution cyprinodont Vg standard solution is the standard solution of 64 ng/mL, carries out 2 double phases dilution of this undiluted solution with a specimen diluent, and prepares each concentration of 32, 16, 8, 4, 2, and 1 ng/mL.

(c) Biotin-ized anti-cyprinodont Vg antibody (x100)

If 30micro (x100) of biotin-ized anti-cyprinodont Vg antibodies L is diluted with specimen diluent 3mL, the biotin-ized anti-cyprinodont Vg antibody solution for 48 wells can be prepared.

(d) HRP-streptoavidin (x500)

If P-SUTORE HR PUTOABIJIN (x500) 6microL is diluted with specimen diluent 3mL, the HRP-streptoavidin solution for 48 wells can be prepared.

(e) Dissolve one lock of OPD locks in substrate liquid 6.5mL returned to the coloring liquid room temperature. (A part for 48 wells)

(**) Return a penetrant remover concentration penetrant remover (x20) to a room temperature, and confirm whether salts have precipitated. Concentration penetrant remover (x20) 30mL is diluted with purified water 570mL, and is used. The diluted penetrant remover is stabilized for 14 days in refrigeration.

[0075] (2) Dilute and use the preparation cyprinodont blood serum of a sample for 10 or more times with a specimen diluent.

[0076] (3) Throw away the penetrant remover in a measurement operating-instructions (b) well, strike a plate on a paper towel etc., often take moisture, and progress to the following step promptly.

(b) Add the standard solution (64 - 0 ng/mL) and a specimen to a well every [50micro / L], and incubate at a room temperature for 2 hours.

(c) Throw away the reaction mixture after reaction termination and in a well, pour a penetrant remover distributively every [at least 300micro / L], and remove by decant. After repeating this actuation twice [further], a plate is struck on a paper towel etc., moisture is often taken, and it progresses to the following step promptly.

(d) Add a biotin-ized anti-cyprinodont Vg antibody to each well every [50micro / L], and incubate at a room temperature for 1 hour.

(e) Perform washing actuation of the ** said appearance after reaction termination.

(**) Add HRP-streptoavidin to each well every [50micro / L], and incubate at a room temperature for 1 hour. At this time, substrate liquid is returned to the room temperature.

Washing actuation of the ** said appearance is performed after (g) reaction termination.

(h) Add coloring liquid to each well every [100micro / L], and make it react for 5 minutes at a room temperature.

(i) Add the reaction stop solution to each well every [50micro / L], and stop an enzyme reaction. It adds systematically like coloring liquid so that the enzyme reaction time amount of each well may become fixed.

(j) Measure 490 or absorbance [of 492nm]" with a plate reader.

(**) Compute the concentration of Cyprinodont Vg from the standard curve shown in drawing 6 .

[0077] [Example 7] Production of an anti-cyprinodont BITEROJIENIN polyclonal antibody (preparation of the immunogen)

1:1 looks 0.6mg of cyprinodont BITEROJIENIN like [Freund's complete adjuvant (FCA : Freund complete adjuvant)] comparatively as a priming antigen, it mixes, a homogeneity emulsion is produced, and it considers as antigen liquid. 1:1 looks 0.2mg of cyprinodont BITEROJIENIN like [the Freund's incomplete adjuvant (FIA : Freund incomplete adjuvant)] comparatively as the 2nd time - 4th immunogen, it mixes,

a homogeneity emulsion is produced, and it considers as antigen liquid.
[0078] (Immunity) It crosses at a time at 1 mL in the regions-of-back hide of a rabbit (Japanese white kind: Japanese White), and crosses to 4 times at intervals of 1 time at two weeks, and dozens of places are inoculated. After the 4th immunity, one week after, from a rabbit, the exsanguination is carried out and anti-cyprinodont BITEROJIENIN antiserum is obtained.
[0079] (Purification of an anti-cyprinodont BITEROJIENIN polyclonal antibody (IgG purification)) Protein equilibrated by PBS G an affinity column (Amersham company) -- anti-cyprinodont BITEROJIENIN antiserum -- in addition, IgG a fraction is adsorbed -- making -- other blood serum components -- PBS It washes. Next, they are 0.1MGlycine(s) about an adsorption fraction. / HC1 pH3.0 It is eluted. Obtained IgG is promptly returned to neutrality and it dialyzes in PBS overnight. Let this be an anti-cyprinodont BITEROJIENIN polyclonal antibody.
[0080]
[Effect of the Invention] (1) The toxic measuring method of the chemical which used this antibody for the antibody which recognizes only BITEROJIENIN of a cyprinodont, and the list by this invention, the evaluation approach of environmental pollution, and the sex judging approach of a cyprinodont are offered.
[0081] (2) It is specific to a cyprinodont, and the antibody of this invention is not influenced of the related protein originating in other ovaries, but exact measurement is possible for it. Therefore, the antibody of this invention is useful to the toxic measuring method of a chemical, the evaluation approach of environmental pollution, and the sex judging approach of a cyprinodont.
[0082] (3) To an environment, it is the so-called sensitive environmental indicator animal, and since the mass production method is possible, the toxicity of a chemical and a cyprinodont's industrial utility value for evaluation of environmental pollution are size in narrow area.

[Translation done.]

* NOTICES *

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2.**** shows the word which can not be translated.
3.In the drawings, any words are not translated.

DESCRIPTION OF DRAWINGS

[Brief Description of the Drawings]
[Drawing 1] It is drawing showing the reactivity of the monoclonal antibody which hybridoma 3C1 produces.
[Drawing 2] It is drawing showing species-specific [of the monoclonal antibody which hybridoma 3H5 produce].
[Drawing 3] It is drawing showing the reactivity over BITEROJIENIN of the cyprinodont in the sandwiches ELISA method of the monoclonal antibody which hybridoma 3C1 produces.
[Drawing 4] It is drawing showing the BITEROJIENIN concentration in the male cyprinodont blood serum which exposed endocrine disruptors into the tank.
[Drawing 5] It is the explanatory view showing the sandwiches ELISA method using a specific antibody in Cyprinodont Vg.
[Drawing 6] It is drawing showing the standard curve of Cyprinodont Vg.

[Translation done.]

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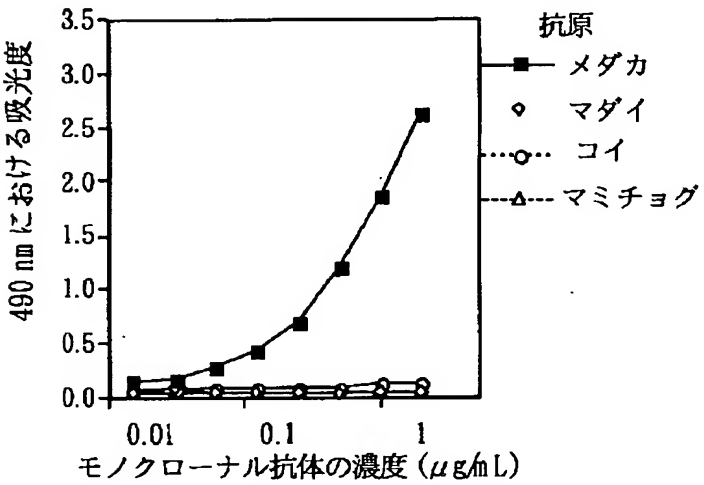
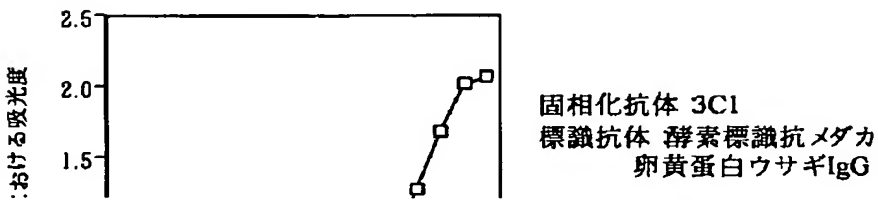
DRAWINGS

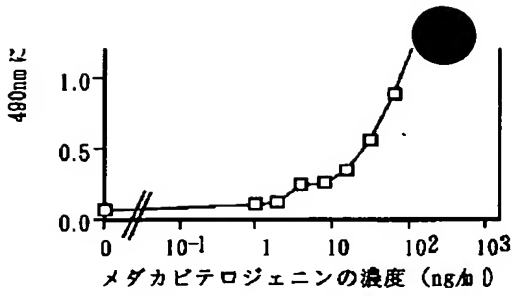
[Drawing 1]
モノクローナル抗体（３Ｃ１）の反応性



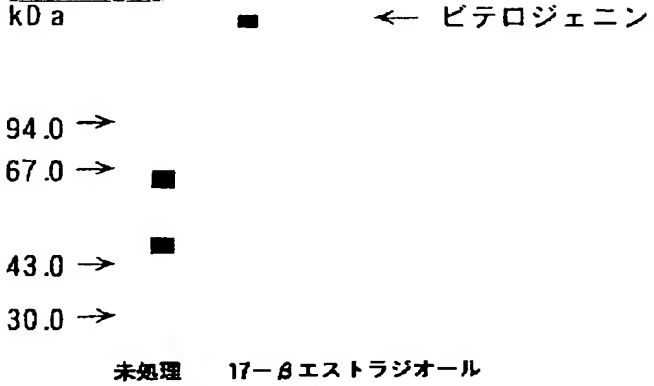
[Drawing 2]
モノクローナル抗体の反応性（３Ｈ５）

[Drawing 3]
サンドイッチELISA法における
メダカのビテロジェニンに対する反応性

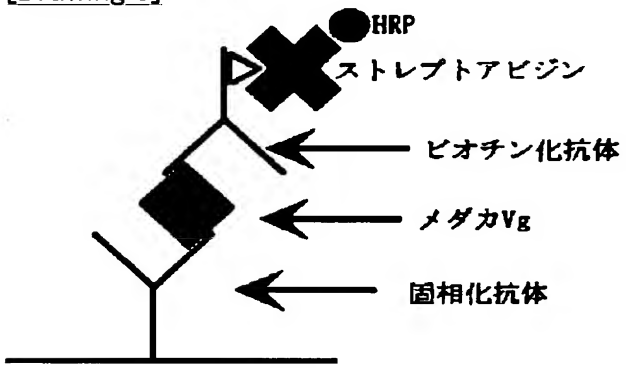




[Drawing 4]

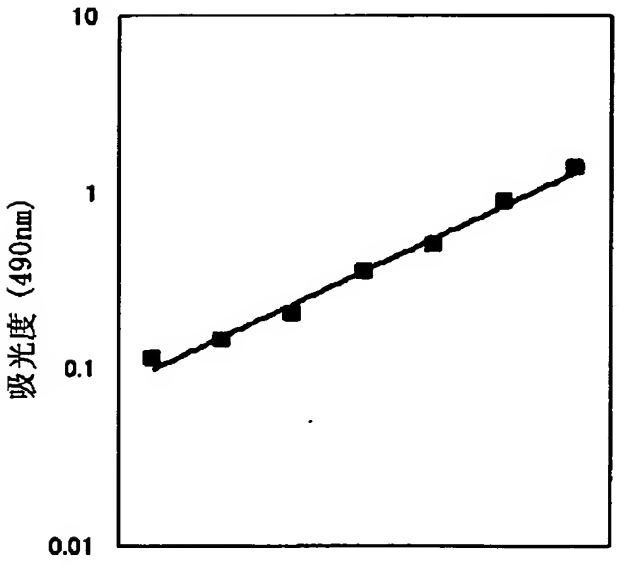


[Drawing 5]



[Drawing 6]

標準曲線



1 2 4 8 16 32 64
メダカピテロジェニン濃度(ng/mL)

[Translation done.]